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We address the mechanisms underlying generation of skeletal muscle, smooth muscle, and endothelium from epithelial progenitors in the dermomyotome. Lineage analysis shows that of all epithelial domains, the lateral region is the most prolific producer of smooth muscle and endothelium. Importantly, individual labeled lateral somitic cells give rise to only endothelial or mural cells (not both), and endothelial and mural cell differentiation is driven by distinct signaling systems. Notch activity is necessary for smooth muscle production while inhibiting striated muscle differentiation, yet it does not affect initial development of endothelial cells. On the other hand, bone morphogenetic protein signaling is required for endothelial cell differentiation and/or migration but inhibits striated muscle differentiation and fails to impact smooth muscle cell production. Hence, although different mechanisms are responsible for smooth muscle and endothelium generation, the choice to become smooth versus striated muscle depends on a single signaling system. Altogether, these findings underscore the spatial and temporal complexity of lineage diversification in an apparently homogeneous epithelium.

Introduction

The somites are epithelial structures arising in a metameric pattern from the paraxial mesoderm. In the course of development, somites undergo successive phases of deepithelialization concomitant with the acquisition of diverse cell fates. Initially, the ventral somite dissociates to generate the sclerotome, which forms the vertebrae, ribs, and tendons. The remaining dorsal part, the dermomyotome (DM), contributes cells to the myotome, the precursor of skeletal muscles and, upon dissociation, also generates the dorsal dermis (Scaal and Christ, 2004).

Previously, we mapped the origin of muscle and dermis from the DM. The initial myotome is established by a population of early specified pioneer myoblasts resident in the medial epithelial somite (Kahane et al., 1998b, 2007). Subsequent myofibers form from all four lips of the DM (Kahane et al., 1998a, 2002; Cinnamon et al., 1999, 2006; Huang and Christ, 2000; Gros et al., 2004), and their proper patterning is determined by the initial scaffold of pioneer fibers (Kahane et al., 2007). In addition to the formation of unit-length myofibers, the DM produces progenitors that remain mitotically active within the myotome (Kahane et al., 2001; Ben-Yair and Kalcheim, 2005) and later develop into either fibers or muscle satellite cells (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). These are generated from the extreme lips of the DM (Kahane et al., 2001) and from the dissociating DM sheet that also produces dermis (Ben-Yair et al., 2003; Ben-Yair and Kalcheim, 2005). Notably, both mitotic myotomal precursors and dermis originate from single cells residing in the central DM sheet. The diversification of these two lineages is accompanied by a striking shift in the plane of epithelial cell division that becomes perpendicular to the mediolateral aspect of the DM. This shift is coupled to the asymmetrical segregation of N-cadherin to the apical daughter cells, which will become muscle, but not to the basal cells, which will give rise to dermis (Ben-Yair and Kalcheim, 2005; Cinnamon et al., 2006).

To better understand the mechanisms responsible for the segregation of the DM epithelium into its derivatives, we turned our attention in this study to the generation of two additional lineages, endothelial and mural cells (vascular smooth muscle and pericytes; Pardanaud et al., 1996; Scaal and Christ, 2004; Esner et al., 2006; Pouget et al., 2006; Wilting and Becker, 2006). The somite-derived endothelium is composed of at least two lineages, blood vessel (BV) cells characterized by the expression

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Abbreviations used in this paper: BMP, bone morphogenetic protein; BV, blood vessel; CV, cardinal vein; DM, dermomyotome; DML, dorsomedial lip; E, embryonic day; HES, hairy and enhancer of split; ICD, intracellular domain; SMA, smooth muscle actin; VEGFr, VEGF receptor; VLL, ventrolateral lip.

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of *VEGF receptor* (*VEGFr*) *2* (*flk-1*) and lymphatic endothelial cells defined by expression of *VEGFr2* and *3* and *Prox-1* (Wilting et al., 2001). *VEGFr2* is expressed in the lateral epithelial somite, and this expression pattern, as well as the normal development of BVs, depends on bone morphogenetic protein (BMP) signaling from the intermediate and lateral plate mesoderm (Nimmagadda et al., 2004, 2005). In vitro studies demonstrated that *VEGFr2*-positive cells can give rise to both endothelium and smooth muscle (Yamashita et al., 2000; Ema et al., 2003). Moreover, *VEGFr2*-positive mouse cells injected into avian embryos generate both lineages in vivo (Yamashita et al., 2000). In contrast, work on transgenic mice carrying the lacZ reporter under the control of the flk-1 promoter demonstrated that in normal development, flk-1–positive cells contribute only to endothelium, hematopoietic cells, and a subset of skeletal muscle but not to mural cells (Motoike et al., 2003). Clonal analysis in avian embryos established that the lateral portion of hindlimb somites harbors progenitors common for both limb endothelial and striated muscle cells (Kardon et al., 2002). Furthermore, recent work demonstrated that mural, endothelial, and skeletal muscle cells share a common lineage (Esner et al., 2006). It is, however, still unclear at which point in development these common lineages diverge and whether they segregate in response to similar or distinct factors.

The possibility that endothelial and mural cells originate from a single DM progenitor seems sensible, as both cell types are of somitic origin and contribute to the walls of the same vessels. Such a scenario would bear resemblance to the segregation of dermal and myotomal fates. Alternatively, these cells may arise from distinct progenitors in the DM. To answer this question, we fate mapped the flank DM epithelium using focal GFP transfection. We find that the lateral portion of the DM is the most productive source for both endothelial and mural cells. In addition, our data indicate that segregation of the two lineages is already underway in the epithelial somite, which is in contrast to myotomal and dermal cells that derive from single progenitors in the central DM sheet.

What is the mechanism underlying the diversification of these three major lineages issued from the lateral DM? One major candidate is the Notch pathway, a well known signaling mechanism that accounts for lineage diversification in many systems (Harris, 1997; Artavanis Tsakonas et al., 1999; Lai, 2004). Notch activity may function to direct arterial-venous specification and arterial differentiation (Domenga et al., 2004) and to regulate endothelial–mural cell interactions during vascular remodelling (Shawber and Kitajewski, 2004). Notch signaling has also been shown to repress muscle differentiation in vitro (Shawber et al., 1996; Wilson-Rawls et al., 1999) and in the avian somite (Hirsinger et al., 2001), as well as to regulate the balance between muscle satellite cells and myofibers at later stages (Conboy and Rando, 2002; Kuang et al., 2007; Vasyutina et al., 2007). Numb functions as an inhibitor of Notch signaling (Guo et al., 1996; Le Borgne, 2006). Recent studies showed that Numb protein is present in the basal pole of epithelial cells comprising the dorsomedial lip (DML) of the DM (Venters and Ordahl, 2005; Holowacz et al., 2006). In myotomal fibers, Numb is distributed homogeneously, and factors that stimulate formation of myofibers increase the levels of Numb protein and promote its homogeneous distribution, further suggesting that Notch signaling is inhibited in muscle cells (Holowacz et al., 2006).

We find that *cNotch2* mRNA is intensely expressed in the entire DM. In addition, *cHairy2*, a member of the hairy and enhancer-of-split (HES) family of transcriptional repressors and Notch effectors (Kageyama et al., 2007), is expressed in the lateral half of the DM. Furthermore, overexpression of constitutively active Notch1 or 2 in the lateral DM significantly biases these progenitors toward a smooth muscle fate, along with a marked increase in expression of both *cHairy2* and smooth muscle actin (SMA). Conversely, inhibition of Notch signaling by overexpression of Numb biases the cells toward a muscle fiber fate at the expense of smooth muscle. Close inspection reveals that production of endothelial cells is less affected by either treatment. Finally, we find that although lateral BMP signaling regulates *VEGFr2* expression and endothelial development, it is not involved in the regulation of *cHairy2* transcription and it does not adversely affect smooth muscle generation. Collectively, these findings suggest that the lateral region of the somite contains separate progenitors for endothelium and smooth muscle whose differentiation is accounted for by distinct signaling mechanisms. In addition, Notch signaling acts antagonistically on the development of smooth versus striated muscle lineages and BMP acts alike on endothelial versus striated muscle fates.

Results

The lateral DML has a major contribution to development of specific BVs

To assess the contribution of the flank-level DM to BV development, we performed lineage analysis of five discrete DM domains, the four extreme lips and the central sheet. Five groups of embryonic day (E)–2.5 (30–32 somite pairs) embryos were injected with GFP-DNA and embryos were further incubated for 40 h until E4. Successful injections gave rise to progeny composed of 1–34 GFP-expressing cells. Results of the lineage analysis are summarized in Fig. 1 A. Of the five domains analyzed, the lateral DM was the greatest source of endothelial and mural lineages (12 and 40%, respectively). The caudal and rostral DM lips contributed to a lesser extent, producing endothelial cells (6.6 and 2.6%, respectively) and mural cells (20 and 18.4%, respectively). The medial DM had a minor contribution to mural cells (5%) and, although the central DM sheet also generated mural derivatives (16.6%), neither the medial DM nor the central sheet produced endothelial cells under these experimental conditions (Fig. 1 A).

Typical locations of endothelial and smooth muscle phenotypes produced by the DM were the cardinal veins (CVs) and vitelline arteries and the mesonephric, dermal, and somatopleural vessels (Fig. 1, C–F; and not depicted). Specifically, lateral DM-derived BV cells were characteristically located in the walls of mesonephric and great vessels, whereas the contribution of the rostral lips and DM sheet was limited almost exclusively to mural cells in dermal BVs. Interestingly, both endothelial and mural cells generated from lateral injections to single somites extended beyond the injected segment to colonize BVs as far as two and one segments away from the site of labeling, respectively; therefore,

A

Domain \ Derivative	Segments with GFP+ cells (%) DM Epithelium	Myotome	Mural cells	Endothelial cells	Dermis/ ID	*n*
Lateral DM E2.5	32	28	40	12	12	25
Medial DM E2.5	40	25	5	0	60	20
Rostral DM E2.5	NA	84.2	18.4	2.6	57.9	38
Caudal DM E2.5	NA	86.6	20	6.6	13.3	15
Central DM sheet E2.5	NA	75	16.6	0	66.6	12

B

Lateral somite E2	18.9	26.4	47.2	22.6	1.9	53
Lateral DM E2.5	32	28	40	12	12	25
Lateral DM E3	0	80	26.6	6.6	20	15

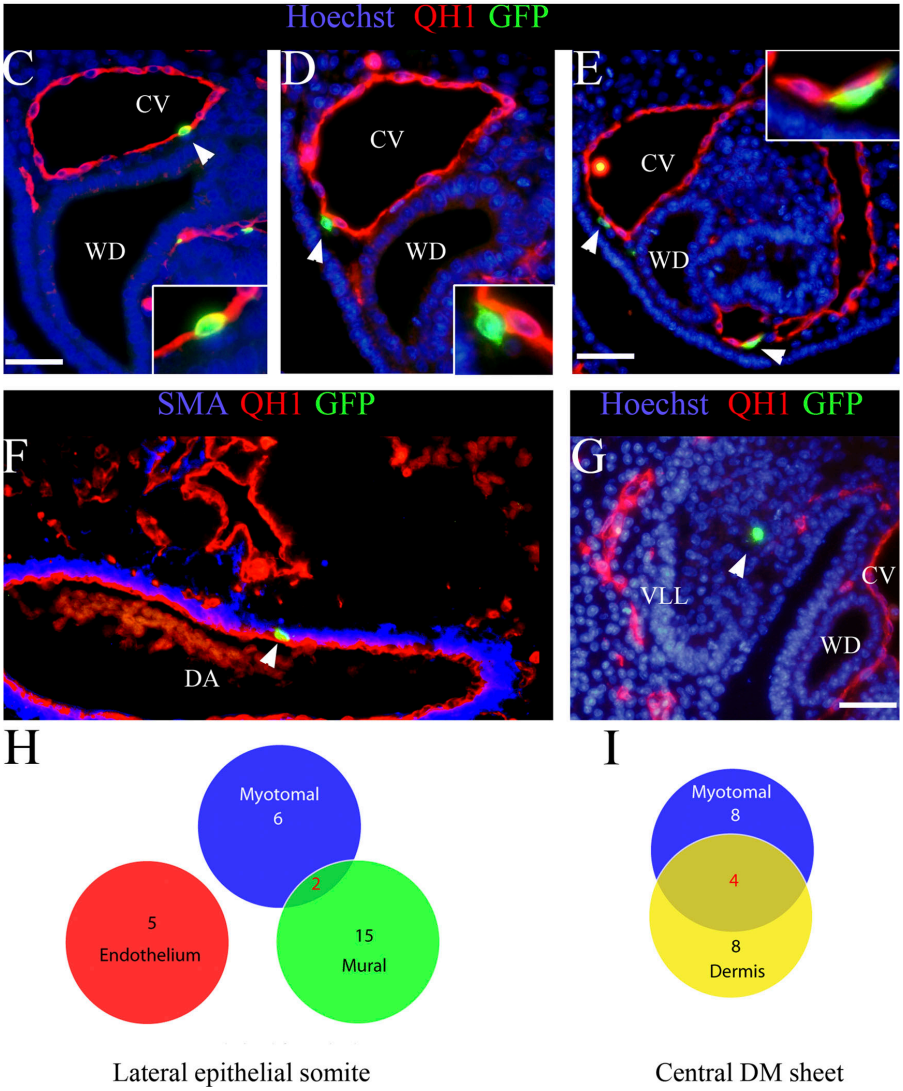

Figure 1. **Lineage analysis of DM domains.** (A and B) Summary of results of GFP injections into five distinct DM domains (A) and into the lateral domain at three different stages (B). Results are expressed as the percentage of segments with labeled cells in the epithelium, in the myotome, in mural and endothelial cells in the wall of BVs, or in the dermis and the intermediate domain (ID). The latter is defined as a DM-derived PAX3/7$^+$ region outside the myotome (Ben-Yair and Kalcheim, 2005). The right column depicts the number of segments analyzed for each domain. NA, not applicable (no epithelium remains in these domains by the time of fixation). (C–G) Typical results 40 h after injections to lateral DMs at E2.5. GFP is green, QH1 is red, and HOECHST nuclear staining is blue, except for F in which SMA immunoreactivity product is blue. (C) QH1-expressing endothelial cell in the wall of the CV (arrowhead, enlarged in inset). Another endothelial cell is located in the wall of a mesonephric vessel. (D and E) Mural cells (arrowheads and enlargements in insets) in the walls of the CV and the mesonephric vessel (E). Note that mural cells are clearly located immediately outside and in contact with the QH1 domain in contrast to GFP$^+$ cells that overlap the QH1 domain (C) and are therefore defined as endothelial cells. (F) SMA-expressing mural cell (arrowhead) in the vitelline artery near the branching from the dorsal aorta (DA). (G) GFP$^+$ cell in the myotome (arrowhead), which is identified as a fiber according to appearance in serial sections. (H and I) Venn diagrams showing the overlap between derivatives in cases arising from selected injections summarized in A and B. (H) Summary of injections to the lateral domain of single E2 epithelial somites/embryos. Mural and endothelial derivatives were never detected in a single embryo, whereas mural and myotomal were occasionally detected together. (I) In contrast, central DM sheet injections at E2.5 very often produced progeny composed of at least dermal and myotomal cells (Ben-Yair and Kalcheim, 2005). WD, Wolffian duct. Bars: (C and D) 30 μm; (E) 39 μm; (F and G) 42 μm.

in contrast to myotomal cells that display a segmentally restricted behavior, these cells visibly migrate along the rostrocaudal axis (unpublished data). It is also worth mentioning that both rostral and caudal DM lips, as well as the central DM sheet, were found to be the major contributors to myotome development (fibers and myoblasts altogether) when compared with the medial and lateral domains (Fig.1, A and G), which is consistent with previous findings (Kahane et al., 1998a, 2002; Ben-Yair and Kalcheim, 2005). Moreover, in addition to the known contribution of the central DM sheet to dermis (Ben-Yair et al., 2003), which was further substantiated here, the medial DM (Olivera-Martinez et al., 2002) and rostral lip also significantly contributed to dermis. The latter source was not recognized previously (Fig. 1 A).

These results show for the first time a direct contribution of the DM epithelium to the smooth muscle lineage. In addition, they point to a differential contribution of DM domains to both BV lineages and to the colonization of specific vessels. For this reason, they raise basic questions regarding the mechanisms of

cell diversification in the DM. To begin exploring these issues, we focused on the contribution of the most productive DM domain in terms of BV lineages, the lateral DM. First, we asked whether its contribution to BVs changes during development. To this end, the lateral portions of epithelial somites or DMs were focally labeled with GFP at E2 (25 somite pairs) and E3 (43–45 somite pairs). 40 h later, successful injections gave rise to 2–8 or 1–31 GFP-expressing cells per segment, respectively. Production of endothelial cells was maximal in the lateral epithelial somite (E2, 22.6%) and progressively diminished to 12% by E2.5 and to 6.6% by E3. The proportion of mural cells was highest at E2 (47.2%) and 2.5 (40%) when compared with that at E3 (26.6%). Notably, late lateral DM (E3) injections produced mostly myotomal derivatives (80%; Fig. 1 B). These findings were further supported by results of more extensive lateral labelings performed by electroporation at E2 or 2.5. The latter produced visibly higher proportions of myotomal cells (mostly fibers) than E2 electroporations, which gave rise to a mixed population of myotomal and BV cells (unpublished data). These results suggest an ordered time course of lineage segregation from the lateral portions of the somite and subsequent DM.

Early segregation of endothelial, smooth, and striated muscle lineages

To further examine the relationship between the endothelial, smooth, and striated lineages, discrete injections of GFP-encoding DNA (one to two cells transfected per injection) were delivered to the lateral domain of single epithelial somites per embryo. The resulting progeny, containing all labeled cells per embryo whether segmentally restricted or not, was analyzed 2 d later (labeled cells were observed in 26 out of 223 embryos with a mean of 12, 3, and 3.8 smooth muscle, endothelial, and myotomal cells per injection, respectively). In 20 out of 26 cases, labeled cells were in BVs. Notably, these BVs contained either GFP^+ endothelial cells or smooth muscle cells but not both, suggesting they derived from distinct progenitors ($P < 0.0001$; Fig.1 H). Mural and myotomal cells were, however, occasionally detected in the progeny of single injections (Fig. 1 H). In contrast, a 50% overlap between dermal and myotomal populations was observed in the progeny derived from the central DM sheet, which is consistent with previous clonal analysis (Ben-Yair and Kalcheim, 2005; Fig. 1 I). Collectively, these data strongly suggest that in the lateral domain, endothelial and mural lineages have begun to segregate from a common precursor by the time of somite formation.

Notch signaling plays an essential role in the choice between mural and myotomal fates but not in initial development of endothelial cells

Notch signaling in the DM. A major question arising from our lineage analysis studies is how cells in the lateral somite and DM give rise to three separate lineages (muscle, mural, and endothelial cells). We examined the possibility that Notch signaling governs cell fate decisions in the lateral DM. Several components of the Notch pathway were shown to be expressed in the DM. The receptor cNotch1 is expressed at low levels throughout the DM (Hirsinger et al., 2001). One of its ligands, cDelta-1, is expressed in the caudolateral corner of the DM. Another ligand, cSerrate-2, is expressed by myotomal cells (Hirsinger et al., 2001; Holowacz et al., 2006).

To complete this picture, we performed in situ hybridizations for additional Notch signaling components. cNotch2 is expressed at high levels throughout the DM (Fig. 2 A). Members of the HES family of transcriptional repressors are well known as direct mediators of Notch signaling (Kageyama et al., 2007). We therefore performed in situ hybridizations with probes for five HES family members. One of the chicken *HES-1* homologues, *cHairy2*, is strongly expressed in the lateral half of the DM (Fig. 2 B), whereas another homologue, *cHairy1*, is not expressed by DM cells but is expressed in the lateral sclerotome (not depicted). Three additional HES family members were detected mainly in the neural tube (*HES-5*, *HES-5/7*, and *HES-6*). *HES-6* appeared in sporadic DML cells only at late stages of DM development (unpublished data). The expression of *cHairy2* indicates that Notch signaling is active in lateral DM cells. Along this line, overexpression of constitutively active Notch1 (N1–intracellular domain [ICD])/GFP or Notch2 (N2-ICD)/GFP, but not of control GFP, strongly up-regulated *cHairy2* mRNA ($n = 16$ and 5, respectively; Fig. 2, C and D; and not depicted). We therefore proceeded to explore the biological roles of Notch signaling in this domain.

Activation of Notch signaling biases lateral DM cells to a mural fate. Constitutively active Notch1 (N1-ICD) or Notch2 (N2-ICD) constructs were coelectroporated with a GFP-encoding plasmid into the lateral DM and embryos were incubated for an additional 40 h. Electroporation of either Notch construct increased the proportions of mural cells out of total GFP^+ cells by threefold when compared with control GFP ($n = 4$ embryos out of 20 with a similar phenotype; Fig. 3, A–C and E–G, compare B and C with E–G). Many Notch-overexpressing cells were located in the walls of the great vessels, especially in the CV, causing a small but consistent increase in SMA/desmin staining in the vein on the treated compared with the untreated side or to control GFP-treated segments (Fig. 3, B, C, and E–G). Interestingly, many Notch-overexpressing cells that stained positive for SMA and desmin were apparent between the ventrolateral lip (VLL) of the DM and the CV. This feature was not detected under control conditions although, occasionally, a few control GFP-expressing cells lacking smooth muscle markers were visible at this location (Fig. 3, E–G, insets). We suggest that these are migrating cells en route to the CV and that Notch signaling strikingly increases their number while prematurely inducing expression of smooth muscle markers. Consistent with the latter notion, premature expression of SMA was already detected around BVs 16–20 h after N2-ICD transfection, which is before the onset of normal expression of this marker under control conditions (Fig. 4, A and B).

Reciprocally, two- and threefold reductions in the proportion of myotomal cells was monitored upon overexpression of N2-ICD and N1-ICD, respectively (Fig. 3, A–C). This effect was visible even in whole embryos observed under a fluorescent binocular. In addition, fewer cells were detected in the residual VLL epithelium. Notably, similar results were already evident 16 h after transfection (unpublished data). Hence, the most significant

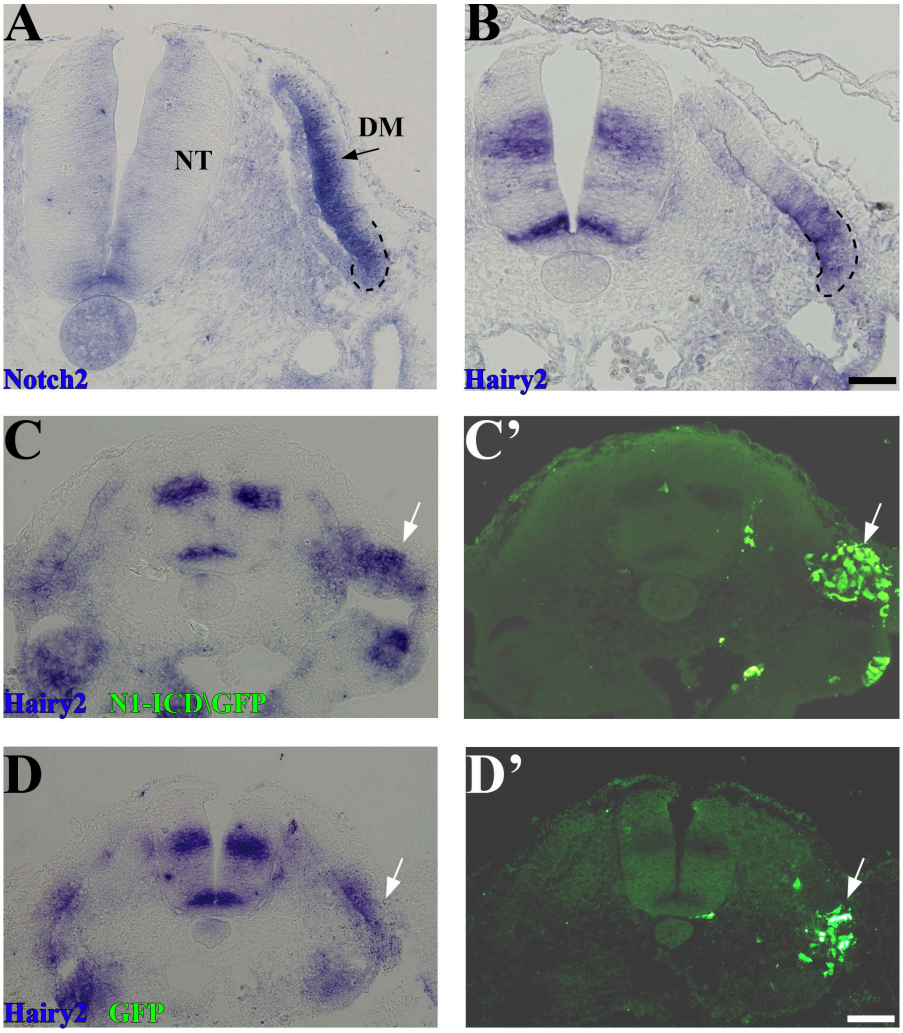

Figure 2. **Expression of Notch signaling genes in the DM.** (A) In situ hybridization with a cNotch2 probe. *Notch2* is expressed throughout the DM epithelium. (B) *cHairy2* mRNA is expressed in the lateral half of the DM. (C and D) *cHairy2* mRNA levels are increased in DMs electroporated with an activated Notch1 construct (arrows in C and C'). Compare with control side and with GFP-electroporated DM (arrows in D and D'). Similar results were obtained in DMs electroporated with activated Notch2. Dashed lines in A and B depict the ventral limit of the DM. NT, neural tube. Bars: (A) 37 µm; (B) 40 µm; (C and D) 114 µm.

effect of Notch activity was enhancing mural cell differentiation at the expense of myotomal fates.

Inhibition of Notch signaling biases lateral DM cells toward a myofiber fate. Electroporation of a Numb construct was used to inhibit endogenous Notch signaling in the lateral DM. Transfected cells were detected using a Numb antibody. Numb-transfected cells contributed almost exclusively to the myotomal domain that contained 88% of the transfected cells, in comparison with 38% in controls ($n = 7$ embryos counted out of 15 with a similar phenotype; Fig. 3 A, B, and D, compare A with D and E; and not depicted). Serial section analysis revealed that these cells were myofibers. In contrast, the contribution to mural cells dramatically decreased from 21.4% of cells in controls to 1.4% in Numb-transfected cells. These effects were already apparent 16 h after transfection (unpublished data). Collectively, both gain- and loss-of-function data suggest that Notch signaling plays a physiological role in the choice between smooth and striated muscle fates from the lateral DM.

In contrast to the antagonistic effects of Notch gain and loss of function on smooth and striated muscle fates, the proportion of endothelial cells decreased regardless of whether Notch activity was up-regulated or inhibited (Fig. 3 A). Yet expression of *VEGFr2* mRNA, an early marker of angioblasts and subsequently of endothelial cells (Wilting et al., 1997), was neither up-regulated nor down-regulated by Notch misexpression (Fig. 4, C and D). Collectively, we suggest that Notch signaling primarily affects the balance between production of mural and myotomal cells but has no direct effect on the acquisition of an endothelial fate. Hence, the reductions observed upon overexpression of either of these factors might result from secondary effects on proliferation rates or survival of endothelial cells.

Different factors regulate the production of endothelial and mural cells from the lateral DM

Production of endothelial cells by the lateral somite and DM is correlated with the dynamic expression of VEGFr2. *VEGFr2* was shown to be expressed in the lateral epithelial somite (Eichmann et al., 1993; Nimmagadda et al., 2004). We thus examined the dynamics of *VEGFr2* expression in relation with results of our fate map analysis (Fig. 1 B). At E2, expression was detected in a broad lateral cluster of epithelial cells adjacent to the intermediate mesoderm (Fig. 5 A, arrow). At E2.5, expression in the DM was apparent in a few VLL cells residing in the extreme portion of the epithelium

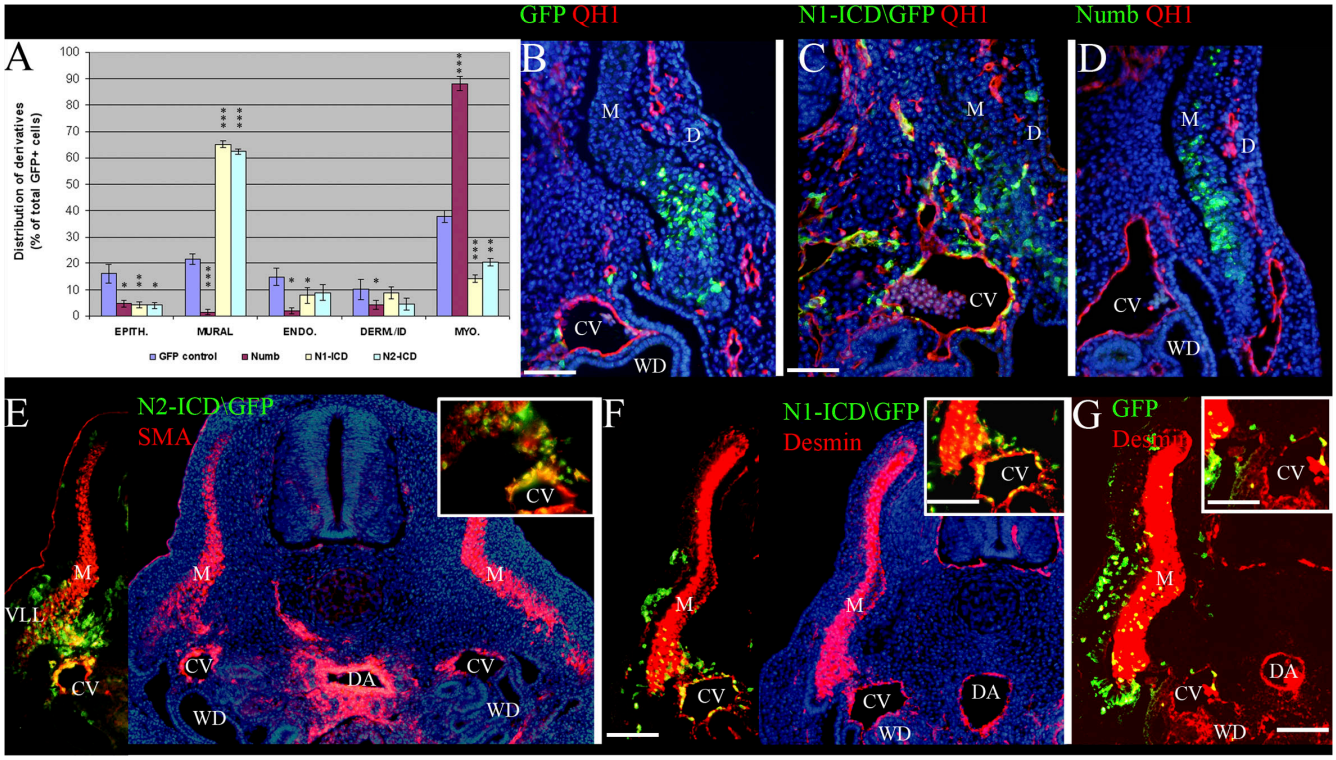

Figure 3. **Notch signaling affects the balance between myotomal and mural cells derived from the lateral DM.** (A) Histogram summarizing the relative phenotypic distribution of labeled cells derived from lateral DMs that were electroporated with either GFP, N1-ICD, N2-ICD, or Numb, 40 h after transfection. Notch overactivation biases cells to a mural fate at the expense of myotomal cells, whereas Numb biases cells to a myotomal fate at the expense of other fates. Results represent mean ± SEM. Significance of results of the different treatments was examined vis-a-vis GFP controls (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (B–D) Electroporations of control GFP (B), N1-ICD/GFP (C), or Numb (D) to lateral E2.5 DMs. GFP or Numb immunoreactivity is green, QH1 is red, and HOECHST is blue. (B) In control GFP-treated epithelium, myotomal and dermal cells are produced along with mural and endothelial cells, which are seen in the wall of the CV. (C) Notch1 biases cells to a mural fate (see E–G). Note the presence of many $GFP^+/QH1^-$ cells in the walls of BVs. Similar results were obtained with N2-ICD. (D) $Numb^+$ cells are predominantly localized in the myotome. (E–G) N2-ICD, N1-ICD, or GFP electroporations to lateral E2.5 DMs. SMA is red in E and desmin is red in F and G. HOECHST is blue. (E) N2-ICD electroporation enhances SMA expression on the treated side and also stimulates the number of GFP^+/SMA^+ cells in the wall of the CV and between the VLL and CV. This is also apparent in the enlarged image appearing in the inset. (F) N1-ICD had a similar effect as revealed by desmin immunostaining. (G) GFP electroporation does not increase desmin staining in the CV wall or between the CV and VLL. WD, Wolffian duct. Bars: (B) 26 µm; (C and D) 60 µm; (E and G) 81 µm; (F) 74 µm; (E and G, inset) 55 µm; (F, inset) 65 µm.

(Fig. 5 B, arrow). At E3, few or virtually no $VEGFr2^+$ epithelial cells could be detected (Fig. 5 C). This progressive decrease in epithelial expression of *VEGFr2* is accompanied by an increasing number of $VEGFr2^+$ endothelial cells already located in the walls of BVs (Fig. 5). This dynamic pattern correlates with the observed time-dependent contribution of the lateral somite and DM to endothelial cells (Fig. 1 B) and further suggests that $VEGFr2^+$ cells in the somite and DM are progenitors of endothelial cells, which progressively delaminate and migrate to colonize the walls of nascent BVs.

BMP signaling is required for differentiation and/or migration of endothelial, but not of mural, cells. BMP was shown to drive the differentiation of endothelial cells in vitro from embryonic stem cells (Park et al., 2004) and in vivo from the lateral somite (Nimmagadda et al., 2004, 2005). We asked whether it is also important for proper differentiation of the mural lineage. First, we examined whether inhibiting BMP signaling had any effect on expression of *hairy2* mRNA. Hairy2 is a transducer of Notch signaling whose mRNA level is stimulated by Notch overexpression (Fig. 2, C and D), an effect accompanied by enhanced mural cell development (Fig. 3). Noggin-DNA was electroporated into the lateral somite of E2 embryos. 12 h after transfection, Noggin-expressing segments exhibited no change in *hairy2* levels, suggesting that BMP is not required for mural cell differentiation ($n = 5$; Fig. 6, A and A'). In contrast, electroporation of noggin-DNA into the lateral mesoderm opposite the segmental plate of E1.5 embryos revealed, 12 h later, a marked reduction of *VEGFr2* expression already in the lateral epithelial somite where its expression is maximal ($n = 8$; Fig. 5 and Fig. 6 B), confirming that BMP is required early during endothelial development.

To directly assess the effects of endogenous BMP on mural versus endothelial cell differentiation, noggin-DNA was electroporated, along with GFP, into the lateral region of flank epithelial somites and embryos were reincubated for 16 h to minimize possible long-term effects. Inhibition of BMP activity resulted in a twofold reduction in the proportion of labeled cells in the myotome, which is consistent with the reported effect of noggin on premature fiber differentiation at the expense of continuous proliferation of lateral progenitors (Kahane et al., 2007). In contrast, it had no significant effect on the proportion of total endothelial or mural cells or of labeled cells remaining in the DM epithelium when compared with GFP controls ($n = 4$ for noggin and control GFP, respectively; Fig. 6 C). Close inspection of serial sections

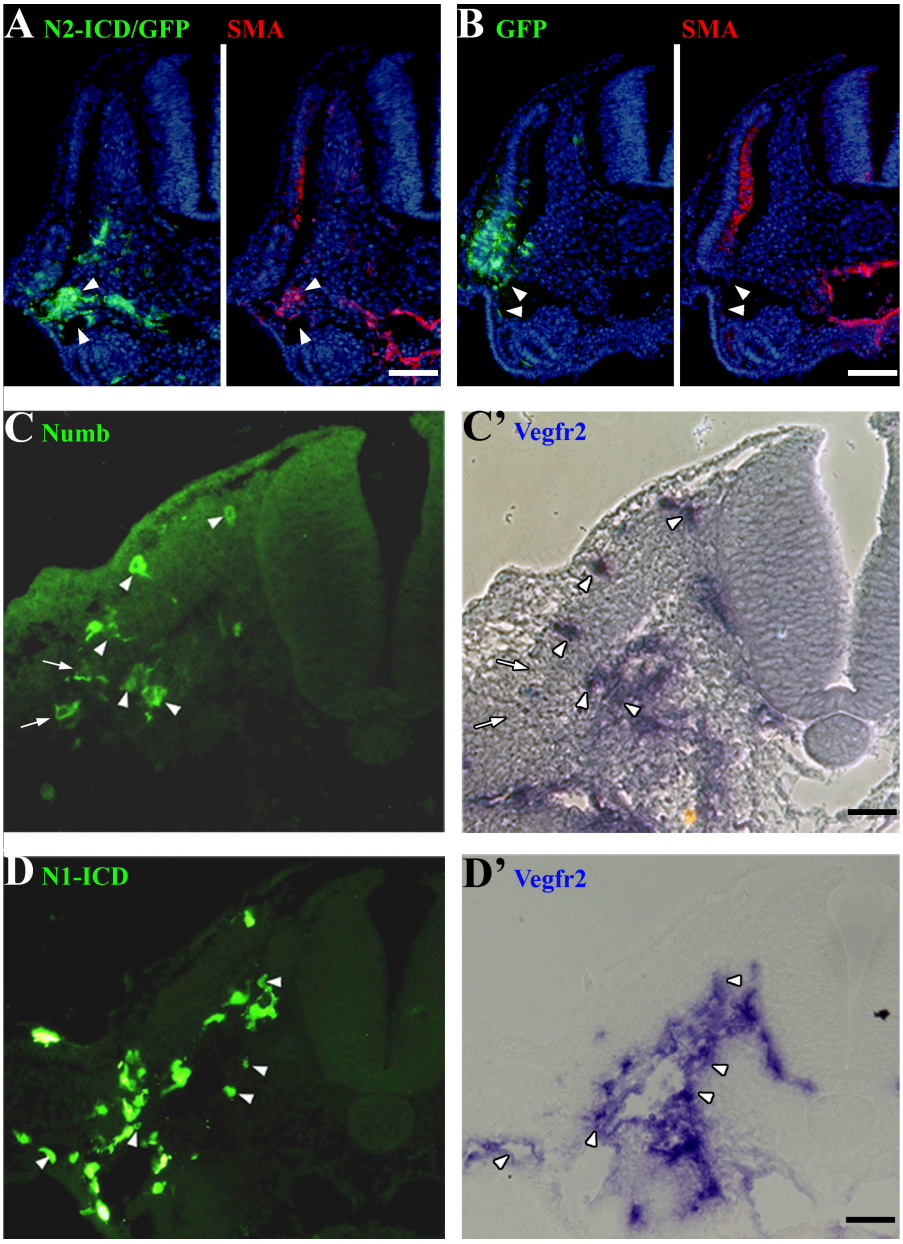

Figure 4. **Notch misexpression causes premature up-regulation of SMA but has no effect on *VEGFr2* mRNA.** Electroporation of N1-ICD/GFP (A, A′, D, and D′), Numb (C and C′), or GFP (B and B′) into lateral somites at E2 followed by fixation 16–20 h later. GFP is green, SMA is red, and HOECHST is blue. (A, A′, B, and B′) Electroporation of N2-ICD/GFP (A) causes premature expression of SMA in the wall of the CV (A′) when compared with control GFP (B and B′). Transfected cells in the wall of the CV are marked by arrowheads. (C and C′) Transverse section at an intersomitic level showing Numb$^+$/*VEGFr2*$^+$ endothelial progenitors (arrowheads) and Numb$^+$/*VEGFr2*$^-$ cells (arrows). (D and D′) Transverse section at an intersomitic level showing N1-ICD$^+$/*VEGFr2*$^+$ endothelial progenitors (arrowheads). Note the large amount of *VEGFr2*$^+$ cells apparent at intersegmental regions. Bars: (A) 54 µm; (B) 68 µm; (C and C′) 31 µm; (D and D′) 44 µm.

revealed, however, that in spite of not affecting the proportion of total QH1$^+$/GFP$^+$ endothelial cells, the latter remained preferentially within the DM epithelium and failed to migrate toward the nascent BVs (Fig. 6, D, E, and G). To get further insight into a possible function of BMP on cell migration and homing to BVs, we focused our analysis on the colonization of the CV (see Materials and methods), a vessel which receives a significant contribution of cells from the lateral somite and DM (Fig. 3). Accordingly, a marked decrease in CV diameter was observed in noggin-electroporated embryos when compared with GFP controls (n = 9; Fig. 6, F and G). We then asked whether the ratio of mural to endothelial cells in this vessel changes upon inhibition of BMP activity. Noggin stimulated by twofold the ratio of mural to endothelial cells in the wall of the CV (from 1.24 mural cells per endothelial cell in embryos that received control GFP to 2.46 mural cells per endothelial cell in noggin-electroporated embryos; n = 9 and 10, respectively; Fig. 6 H). Because noggin does not affect the proportion of mural cells or the expression of *hairy2* but severely down-regulates *VEGFr2* and the ability of endothelial progenitors to delaminate from the epithelium and/or to migrate, we conclude that the increased proportion of mural/endothelial cells is accounted for by a decrease in the contribution of the DM to endothelial cells. Collectively, these data suggest that lateral BMP is required for the proper differentiation and/or migration of endothelial, but not of mural, cells.

Discussion

The DM, although displaying an overall homogeneous pattern of growth (Ben-Yair et al., 2003), cannot be regarded as a uniform epithelium. Rather, it is composed of subdomains distinguished by gene expression patterns and generation of various cell fates

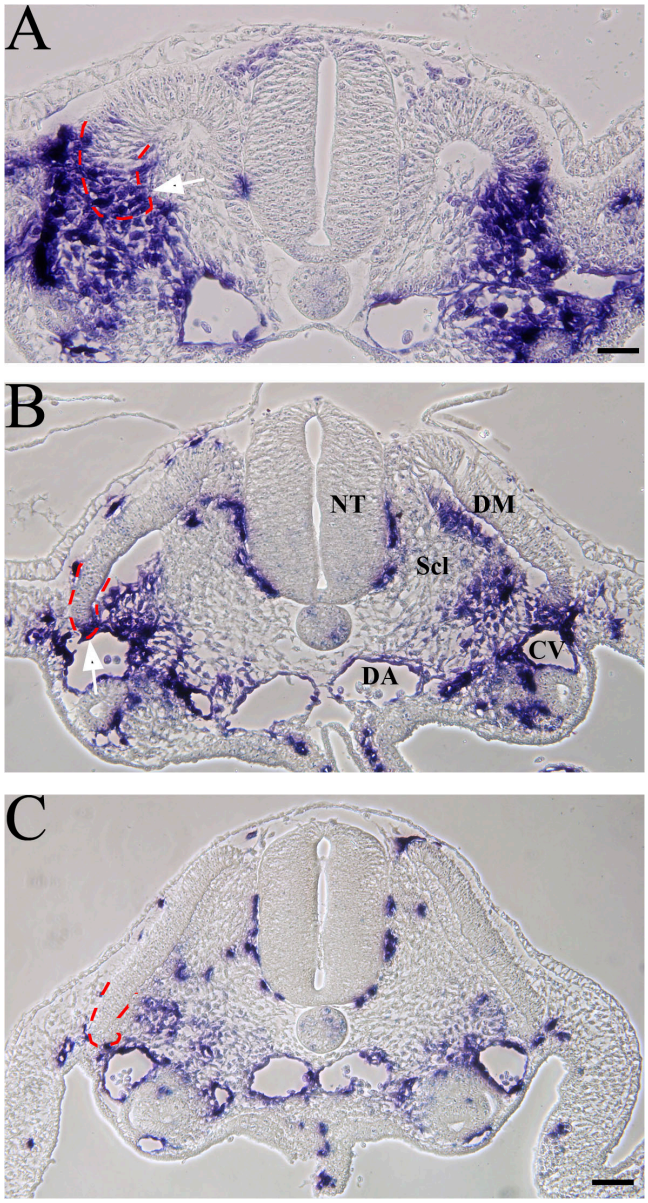

Figure 5. **Dynamics of *VEGFr2* expression in the lateral somite and DM.** (A) Strong expression is detected in progenitors residing in the lateral region of flank-level epithelial somites (arrow). (B) Few cells expressing *VEGFr2* are apparent in the lateral-most epithelium of E2.5 flank-level DMs (arrow). (C) By E3, the DM epithelium is VEGFr2 negative. The lateral border of the somite–DM epithelium is marked by a red dashed line. DA, dorsal aorta; NT, neural tube; Scl, sclerotome. Bars: (A) 17 µm; (B and C) 35 µm.

(Pardanaud et al., 1996; Scaal and Christ, 2004; Kalcheim and Ben-Yair, 2005; Esner et al., 2006). This motivated us to investigate the mechanisms underlying the development of mural and endothelial cells that contribute to the walls of BVs in comparison to myotomal cells. The first part of this study entailed a comprehensive lineage analysis of DM subdomains, which established that in the somite and early DM, the lateral domain is the major contributor of both endothelial and mural cells. In addition to unraveling a direct derivation of smooth muscle cells from the DM, these experiments reveal a time-dependent generation of cell fates from the lateral domain and suggest that mural and endothelial cells arise from distinct somitic progenitors with endothelial cells being an early segregated population. In addition, because the relative level of endothelial cell production drops first, in association with the disappearance of *VEGFr*$^+$ cells from the lateral DM, we suggest that in addition to being spatially limited, the pool of endothelial precursors is finite and temporally restricted.

The second part of this study demonstrates that Notch signaling plays a physiological role in the generation of smooth versus striated muscle from the lateral DM and has little if any effect on initial specification of the endothelium. Reciprocally, BMP signaling, despite its effect on the differentiation and/or migration of somite-derived endothelial cells via regulation of *VEGFr2* expression, does not seem to affect smooth muscle development yet inhibits differentiation of myotomal myofibers (Pourquie et al., 1996). Thus, we suggest that initial development of mural and endothelial cells is governed by different mechanisms. In contrast, development of mural and striated muscle sublineages is a binary choice that depends upon a single signaling system (Fig. 7).

The DM origin of cells that contribute to the walls of BVs

The contribution of the lateral somite to endothelial and mural lineages has already been reported (Eichmann et al., 1993; Pardanaud et al., 1996; Kardon et al., 2002; Scaal and Christ, 2004; Esner et al., 2006; Wiegreffe et al., 2007); however, a comprehensive fate map of the DM contribution to these lineages has not been available until now. Although a DM origin of endothelial cells was established (Scaal and Christ, 2004), we report for the first time that the DM is also a direct source of smooth muscle. Moreover, we find that endothelial and mural cell production is not entirely restricted to the lateral DM. To a smaller extent, endothelial cells are also produced by rostral and caudal lips but not by the DM sheet or DML. Mural cells are generated from all DM domains but much more so from the lateral region. Notably, mural cells stemming from different DM domains contributed to distinct vessels in a stereotypic fashion.

When the composition of GFP-expressing cells is considered, a surprising aspect of endothelial and mural cell differentiation is revealed: most injections gave rise to only one type of derivative, even though injections were performed as early as the epithelial somite stage. This result raises the possibility that the progenitors at the origin of these two lineages are fate restricted already at the epithelial somite stage, being able to generate, e.g., smooth and striated muscle but not endothelium and smooth muscle. This does not exclude the option that bipotent progenitors may still exist at this stage; however, our data would argue that they are a minor subset. In contrast to the separation between mural and endothelial lineages, we found many cases containing both dermis and myotomal cells (Ben-Yair and Kalcheim, 2005) and also additional phenotypic combinations. Collectively, we sustain that the observed absence of cases with both endothelium and smooth muscle is of physiological significance. In spite of a putative fate restriction, these progenitors are not necessarily fully or irreversibly committed. In fact, our experimental data show that we can still affect lineage decisions between smooth and striated muscle and between striated muscle and endothelium.

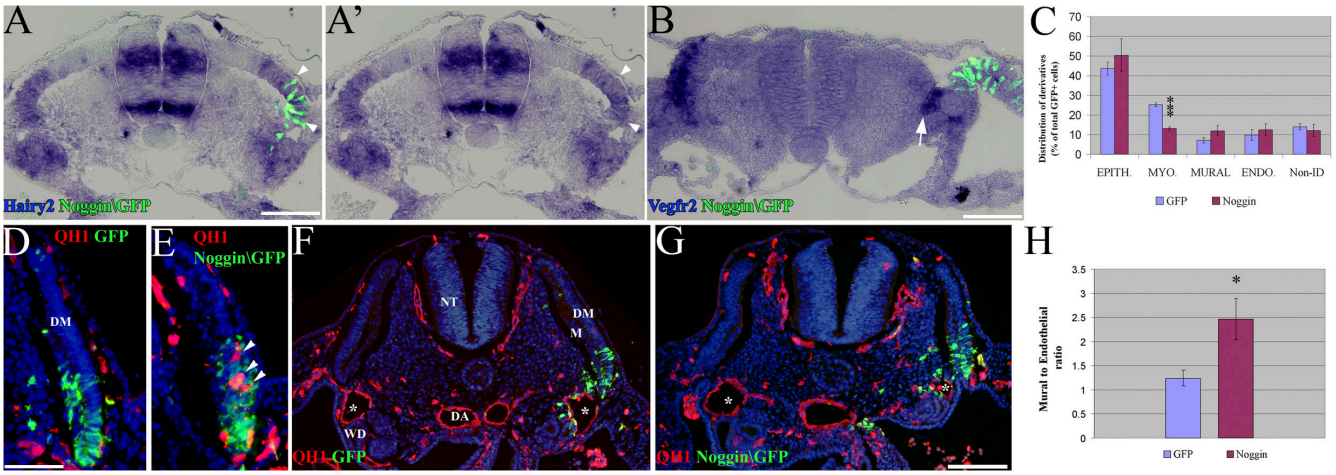

Figure 6. **Inhibition of BMP activity by Noggin affects endothelial but not smooth muscle development.** (A and A') Noggin electroporation into the lateral epithelial somite does not affect *cHairy2* expression. Arrowheads delimit the electroporated area. (B) Noggin electroporation into intermediate and lateral plate mesoderms severely inhibits *VEGFr2* expression in the lateral somite (arrow). A similar effect was observed upon electroporation of the lateral somite (not depicted). (C) Noggin electroporation into lateral somites reduces the proportion of labeled cells in the myotome 16 h after treatment, compared with control GFP-electroporated somites ($P < 0.001$), but does not change the proportions of mural or endothelial cells. Results represent mean ± SEM. (D and E) Noggin electroporation (E) into lateral somites alters the differentiation/migration pattern of endothelial cells when compared with control GFP (D). Many QH1-positive cells remain atypically located within the DM epithelium in noggin-treated DMs (arrowheads in E; see G) rather than homing to nascent BVs. (F and G) Lateral somites of E2 embryos were electroporated with Noggin-GFP (G) or control GFP (F). 16 h after treatment, the CV (asterisks) on the treated side exhibits a significant reduction in lumen diameter when compared to the untreated side or to GFP electroporation. Noggin/GFP cells are green. (H) Noggin increases the ratio of mural to endothelial cells in the CV 16 h after electroporation into lateral E2 somites ($P = 0.02$). Results represent mean ± SEM. QH1 is red and HOECHST is blue in D and E. DA, dorsal aorta; M, myotome; NT, neural tube; WD, Wolffian duct. Bars: (A and A') 53 µm; (B) 50 µm; (D and E) 43 µm; (F and G) 120 µm.

Nevertheless, endothelium and smooth muscle appear to be differentially affected by BMP and Notch, respectively, further validating the notion that their respective progenitors are already restricted in their developmental potential. Moreover, the transient expression of *VEGFr2* in the lateral-most somite and the rapid exhaustion of *VEGFr2*$^+$ endothelial progenitors from the lateral DM favor the view that, at least, the endothelial precursors are an early specified cell population.

Fate analysis of lateral somite cells, performed at hindlimb levels of the axis, proved that a significant proportion of single progenitors produce both endothelial and striated muscle cells (Kardon et al., 2002). Our lateral injections generated, instead, only a small proportion of both endothelial and muscle cells even if injections were not clonal, suggesting that in flank somites, the existence of a common progenitor for the two derivatives is not a common event. Possibly, fate segregation at hindlimb levels is a later process as lateral progenitors delaminate and migrate extensively into the limb before overt differentiation. Retrospective lineage analysis in transgenic mice using the nlacZ reporter showed that endothelial, smooth muscle, and striated muscle cells share a common early progenitor present before somitogenesis (Esner et al., 2006). Our data, stemming from direct labeling of epithelial somites and DM tissue, suggest that the majority of progenitors are already fate restricted at the epithelial somite stage. Collectively, these results would argue that fate restriction begins sometime during the transition from the presomitic to the segmented paraxial mesoderm.

The generation of derivatives from the lateral DM follows a stereotypic temporal pattern with endothelial cells being produced mostly at early stages (somite and early DM). This correlates with the observed pattern of *VEGFr2* expression in the lateral somite and young DM. Nevertheless, mural cell production is significant also at later stages of DM development after *VEGFr2* expression in the DM had disappeared. We therefore reason that *VEGFr2* activity in somites mediates development of endothelial, but not of mural, cells, although we cannot rule out the possibility that some *VEGFr2*-positive cells also contribute to the latter. Our results, stemming from lineage analysis as well as from responsiveness to different signals, are in agreement with results obtained in transgenic mice expressing LacZ under the control of the *VEGFr2* promoter in which lacZ-expressing cells contributed to endothelial but not to mural derivatives (Motoike et al., 2003). VEGFr2-positive cells are capable, however, of also producing mural derivatives under several experimental conditions (Yamashita et al., 2000; Ema et al., 2003).

Notch signaling plays a role in cell fate determination in the lateral DM

We find that overexpression of either Notch1 or 2 strongly promotes the differentiation of mural cells from the DM, which is accompanied by a significant increase in *cHairy2* mRNA and premature expression of SMA protein. Reciprocally, inhibition of endogenous Notch with Numb strongly biased cells to muscle fiber fate at the expense of other fates, particularly smooth muscle. This is consistent with a physiological role for Notch proteins in the binary choice between smooth versus striated muscle fates. Thus, Notch acts not only by inhibiting striated muscle differentiation (Hirsinger et al., 2001), it also positively stimulates differentiation of smooth muscle. Our data are consistent with recent results implicating Notch in differentiation of smooth muscle from embryonic stem cells in vitro (Doi et al., 2006) and from neural crest cells in vivo (High et al., 2007), thus emphasizing the

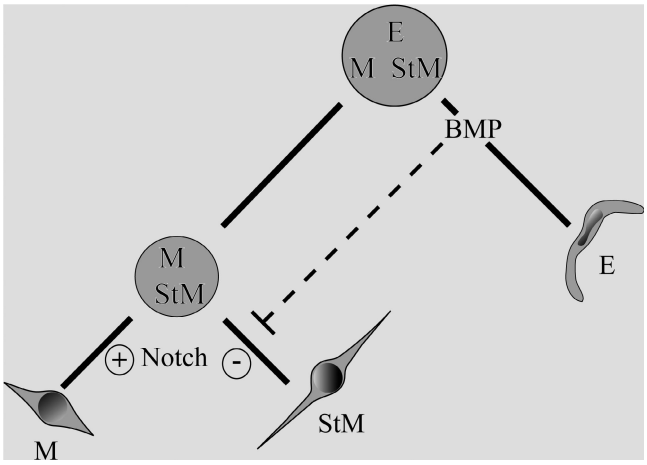

Figure 7. **A simplified model for lineage segregation in the lateral somite/DM.** Endothelial (E), smooth muscle (M), and striated muscle (StM) lineages arise from a common progenitor (Esner et al., 2006). Direct lineage analysis of lateral somite and DM show that fate-restricted progenitors are already detected at the epithelial somite stage. Of the three lineages, endothelial cells constitute a population of early segregated progenitors whose differentiation and/or migration depends on lateral mesoderm-derived BMP. In turn, we postulate the existence of a common intermediate progenitor for smooth and striated muscle sublineages from which separate fates are generated over a more extended period. The segregation of these fates is a binary choice that depends on Notch signaling. Elevated activity of Notch stimulates smooth muscle development, whereas lower levels or inhibition of Notch signaling drive generation of striated muscle. Under experimental conditions, abrogation of Notch function by overexpression of Numb results in myofiber differentiation in the myotome at the expense of mitotic muscle progenitors (satellite cell or myofiber progenitors). It is possible, however, that in the embryo, more subtle differences in levels of Notch activity modulate the balance between generating a myofiber or remaining a mitotic muscle precursor within the striated muscle sublineage. Likewise, inhibition of BMP activity was found to stimulate premature MyoD transcription and lateral myofiber differentiation (Kahane et al., 2007) leading to a reduction in cell number within myotomes (Fig. 6). Hence, the development of striated muscle precursors is negatively modulated by Notch as well as by BMP signaling cascades. Phenotypic segregation in the DM, therefore, exemplifies how two signaling cascades produce three distinct lineages by combining agonistic and antagonistic interactions.

significance of Notch signaling as a generic mediator of smooth muscle development from different sources.

Notch signaling has been shown to regulate production of alternative cell fates by lateral inhibition (Chitnis et al., 1995; Bray, 2006). In other situations, dividing cells segregate Numb protein asymmetrically, thereby inhibiting Notch signaling in the daughter cells that inherited Numb, a process that results in production of alternative fates (Rhyu et al., 1994; Guo et al., 1996; Cayouette and Raff, 2003). Because Numb isoforms are known to be expressed in the DM (Venters and Ordahl, 2005; Holowacz et al., 2006), theoretically any one of these two mechanisms could play a role in the choice between striated versus smooth muscle production from lateral DM cells. Our lineage analysis data exclude the possibility that single dividing cells in the lateral DM produce two daughters bearing alternative fates because injections have a strong tendency to give rise to only one of these fates. Yet the possibility remains open that lateral inhibition is operative in this system. Along this line, somite precursors expressing a Notch ligand would become striated muscle, whereas their neighboring cells, in which the Notch pathway is activated, would generate smooth muscle. A third possibility to explain our observations is that Notch has opposite effects on the actual differentiation of progenitors for smooth versus striated muscle. This would be consistent with data suggesting that Notch signaling acts downstream of Myf5 to inhibit muscle differentiation via inhibition of MyoD transcription (Hirsinger et al., 2001) and with additional results suggesting a positive effect of Notch on smooth muscle development (see previous paragraph). In such a case, the responsive progenitors could already be at least partially specified at the epithelial somite stage or, alternatively, bear unique positional information. This would explain why injected cells or cell clusters tend to produce only one fate in our lineage-tracing experiments.

Collectively, the present findings further highlight the spatial and temporal complexity of lineage segregation in the somitic epithelium. Elucidating the precise mechanisms underlying lineage segregation in the various domains of the epithelium will be the focus of future studies.

Materials and methods

Embryos

Fertile quail (*Coturnix coturnix japonica*) eggs from commercial sources (Moshav Mata) were used.

Expression vectors and electroporation

The five expression vectors used were the following: pCAGGS-AFP (Momose et al., 1999); Numb (pMIW-cNumb) and activated cNotch1 (pMIW-CNICΔc89, N1-ICD; obtained from Y. Wakamatsu, Tsukuba University, Ibaraki, Japan; Wakamatsu et al., 1999); mouse activated Notch2 (N2-ICD; obtained from S. Chiba, Tokyo University, Tokyo, Japan; Shimizu et al., 2002); and xNoggin (Endo et al., 2002). The latter two were subcloned into pCAGGS. Electroporations were performed under a dissecting microscope (M8; WILD). 1–4 μg/μl DNA was microinjected into the center of flank-level epithelial somites or young DMs (Ben-Yair et al., 2003). For lateral-plate injections, the DNA was introduced between the two layers of mesoderm. Electrodes were positioned bilaterally in the intraembryonic coelom and parallel to the rostrocaudal axis. A four-parameter PulseAgile square wave electroporator (PA-4000; Cyto Pulse Sciences, Inc.) was used to deliver three groups of sequential pulses: 3 × 30 V, 20 ms each; 1 × 38 V, 5 ms each; and 3 × 30 V, 20 ms each.

Transfection of a GFP-encoding plasmid into discrete DM cell populations: specificity controls

Direct injections of GFP-DNA to somite and DM cells were performed as previously described (Ben-Yair and Kalcheim, 2005). All injections were directed to flank somites 20 through 25. For epithelial somite labelings, embryos aged 25 somites were used, and for DM injections, embryos were 30–32 somites old (E2.5) or ∼43 somites (E3). Although originally developed for clonal transfection, we aimed instead to trace small cell subsets rather than performing clonal analysis. To this end, a concentration of 1 μg/μl DNA was used. Based on the final number of GFP^+ cells monitored at fixation, we estimate that 1–2 cells were labelled/injected.

To assess that injections hit the DM as opposed to the sclerotome, which also produces smooth muscle and endothelium, 40 embryos were injected with GFP-DNA in the lateral DM, the region closest to the sclerotome, and fixed 4 h later. In 34 out of 40 cases, labelled cells were found in the lateral DM and in 1 out of 40 in both the lateral DM and myotome. In addition, in 1 case, the lateral mesoderm was labelled and in 4 out of 40 segments the lateral myotome was attained. No sclerotomal transfection was observed under these conditions, thus validating the specificity of the method (Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200707206/DC1). Likewise, more prominent transfections of GFP-DNA, performed by electroporations to the lateral DM, revealed 4 h later that only this epithelial domain contained labelled cells. Occasionally, a few DM cells were located more medially and, in only one case, a few sclerotomal progenitors were attained (n = 8; Fig. S1 C).

Embryo processing and in situ hybridization

Embryos were fixed with either 4% formaldehyde or Fornoy and processed for paraffin wax embedding (Cinnamon et al., 1999). In situ hybridization was performed with probes for *VEGFr2* (Eichmann et al., 1993), *cHairy2* (obtained from I. Palmeirim, Minho University, Braga, Portugal; Jouve et al., 2000), and *cNotch1* (Myat et al., 1996), as well as with the following probes from the BBSRC ChickEST database (ARK Genomics): two *HES5* homologues (clones ChEST395k9 and ChEST863a6); *HES6* (clone ChEST145f3); and *Notch2* (clones ChEST832m1 and ChEST1007g3). Some sections were further subjected to anti-GFP immunohistochemistry.

Immunohistochemistry and image processing

Immunostaining was performed with polyclonal and monoclonal antibodies to desmin (1:100 [MP Biomedicals]; and 1:20 [Sigma-Aldrich]) that recognize both smooth and striated muscle lineages (Gerhardt and Betsholtz, 2003), polyclonal antibodies to GFP (1:200; Invitrogen), antibodies to quail endothelial marker QH1 (1:10; Developmental Studies Hybridoma Bank), monoclonal antibodies to SMA (1:400; Sigma-Aldrich), and polyclonal antibodies to chicken Numb (1:1,000; Wakamatsu et al., 1999). Secondary antibodies coupled either to Cy2 or Rhodamine were used (1:200; Jackson ImmunoResearch Laboratories). DakoCytomation was used as a mounting medium. Micrographs were taken from a microscope (BX51; Olympus) with U plan FL-N 10×/0.30, 20×/0.5, and 40×/0.75 dry objectives (Olympus) at RT, using DP controller v1.2.1.108 acquisition software (Olympus) coupled to a cooled charge-coupled device digital camera (DP70; Olympus). For figure preparation, images were exported into Photoshop CS2 (Adobe). If necessary, the brightness and contrast were adjusted to the entire image, and images were cropped without color correction adjustments or γ adjustments. Final figures were prepared using Photoshop CS2.

Data analysis

Lineage analysis of DM domains. The fates of distinct DM regions were quantified as previously described (Ben-Yair and Kalcheim, 2005). All sections containing GFP$^+$ cells and visible HOECHST-positive nuclei (ranging between 1 and 15 sections per embryo) were photographed, and phenotypes were classified according to marker expression, relative position, or both. 1–34 cells were detected per segment and 12–28 such cell groups were analyzed per domain. Endothelial cells were identified by expression of QH1, mural cells were identified by either SMA or desmin and by their association with QH1$^+$ cells, and myotomal (fibers and myoblasts) and dermal cells were identified by position (Fig. 1, C–G). Results are expressed as the percentage of segments (somite length units) with GFP$^+$ cells for each derivative. Because some segments contained more than one type of derivative, totals may exceed 100%. This method was found to be more reliable than absolute cell counts because of the impact of differential cell division rates among the different derivatives, which was significant.

For statistical evaluation of data presented in Fig.1 H, the stringent-null hypothesis that progenitors were multipotent at the time of transfection and divided only once before specification was assumed. The sum of differences between the observed and expected values was compared with the χ^2 distribution to determine the goodness of fit of the model.

Electroporations. Cells expressing GFP and cell type–specific markers were counted as described in the previous paragraph. To ensure uniformity, sections spanning several segments with labeled cells were photographed and counted. The proportion of a given derivative was calculated as the number of labeled cells out of the total number of GFP$^+$ cells in all sections and expressed as mean ± SEM. 174–1,101 cells were counted per embryo in quantifications that were made 48 h after treatments, or 24–276 cells were counted per embryo in quantifications made 16 h after treatments. In noggin-electroporated embryos, a range of 296–755 (total GFP$^+$ cells) or 21–188 (GFP$^+$ cells in CV) cells per embryo were counted, and results are expressed as the ratio of labeled derivatives out of the total number of GFP$^+$ cells in all sections or as the ratio of mural to endothelial cells ± SEM, respectively. Significance of the results was determined using the unpaired *t* test.

Online supplemental material

Fig. S1 shows the specificity of GFP-DNA injections (A and B) or electroporation (C) to the lateral DM. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200707206/DC1.

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